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# The Product of the UL12.5 Gene of Herpes Simplex Virus Type 1 Is Not Essential for Lytic Viral Growth and Is Not Specifically Associated with Capsids

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The herpes simplex virus type 1 UL12 gene encodes a pH-dependent deoxyribonuclease termed alkaline nuclease. An N-terminally truncated version of the UL12 gene, called UL12.5, was shown to be translated independently from a subgenic mRNA which shares its 3′ terminus with the full-length UL12 mRNA. We showed previously that the UL12.5 gene product cannot compensate for the absence of the full-length UL12 gene product (R. Martinez, L. Shao, J. C. Bronstein, P. C. Weber, and S. K. Weller, 1996, *Virology* 215, 152–164); however, it was not known whether UL12.5 itself performs an essential function during lytic viral growth. In this article the initiation codon for the UL12.5 gene product was mapped and altered to create a gene no longer capable of producing UL12.5. This mutation was introduced into the viral genome to create a virus which was capable of producing full-length UL12 but not UL12.5. The growth properties of this virus indicate that UL12.5 is not essential for viral growth in culture. UL12.5 was previously reported to represent a capsid-associated form of alkaline nuclease (J. C. Bronstein, S. K. Weller, and P. C. Weber, 1997, *J. Virol.* 71, 3039–3047). Sucrose sedimentation analysis of capsids from cells infected with wild-type or mutant viruses indicates that both UL12 and UL12.5 are found in fractions from across the sucrose gradient which do not always correlate with the presence of viral capsids. Furthermore, UL12.5 is found in fractions across the gradient even in cells infected under conditions in which no capsids are formed. These results indicate that UL12.5 does not specifically associate with viral capsids. Taken together, these data indicate that UL12.5 is not likely to play an important role in lytic viral infection. 

## INTRODUCTION

Herpes simplex virus type 1 (HSV-1) encodes an exonuclease termed alkaline nuclease, the product of the UL12 open reading frame. The UL12 gene represents a 626 amino acid open reading frame predicted to encode a protein with a molecular weight of 67 kDa (McGeoch *et al.,* 1986b, 1988). The alkaline nuclease has been implicated in the processing of DNA replication intermediates during infection (Goldstein and Weller, 1998b; Martinez *et al.,* 1996a). We previously constructed a UL12 *null* mutant (AN-1) in which 917 bp of UL12 sequence was replaced by a *lac*Z insertion (AN-1). AN-1 is severely compromised in the production of progeny virus with yields 0.1–1% that of wild-type virus (Shao *et al.,* 1993). In the absence of UL12, we have observed that DNA-containing capsids are formed; however, they are not transported from the nucleus to the cytoplasm (Shao *et al.,* 1993). Furthermore, increased levels of abortive A capsids accumulate. We have proposed that in the absence of UL12, aberrant genomes are produced and packaged, resulting in the formation of DNA-containing capsids which are not competent to leave the nucleus. Furthermore, empty capsids accumulate presumably due to disgorgement of aberrant genomes.

The following five 3′-coterminal mRNAs have been mapped in the region of the HSV genome responsible for encoding UL12: 4.5, 3.9, 2.3, 1.9, and 0.9 kb (Costa *et al.,* 1983; Draper *et al.,* 1986). The 2.3-kb mRNA encodes UL12 and is expressed as an early $\beta$ message (see Fig. 1). The 4.5-, 3.9-, and 0.9-kb messages encode the products of the UL14, UL13, and UL11 genes, respectively. The 1.9-kb message was predicted to encode an N-terminally truncated version of the UL12 gene, and *in vitro* translation of the 1.9-kb message produces a 54- to 60-kDa protein (Costa *et al.,* 1983). We previously constructed a viral mutant, AN-F1, in which the full-length UL12 open reading frame was interrupted by the insertion of two base pairs 11 bp downstream of the initiation codon of UL12 (Martinez *et al.,* 1996b) (See Fig. 1). The resulting virus, AN-F1, was not able to produce full-length UL12; however, a smaller protein of 55 kDa, which reacts with UL12-specific antisera, was detected. The truncated version of UL12 was named UL12.5, and it was expressed under conditions in which no UL12 was present. This result was interpreted to indicate that it could be produced by an independent mRNA, most likely the 1.9-kb species previously reported by Costa *et al.* (Costa

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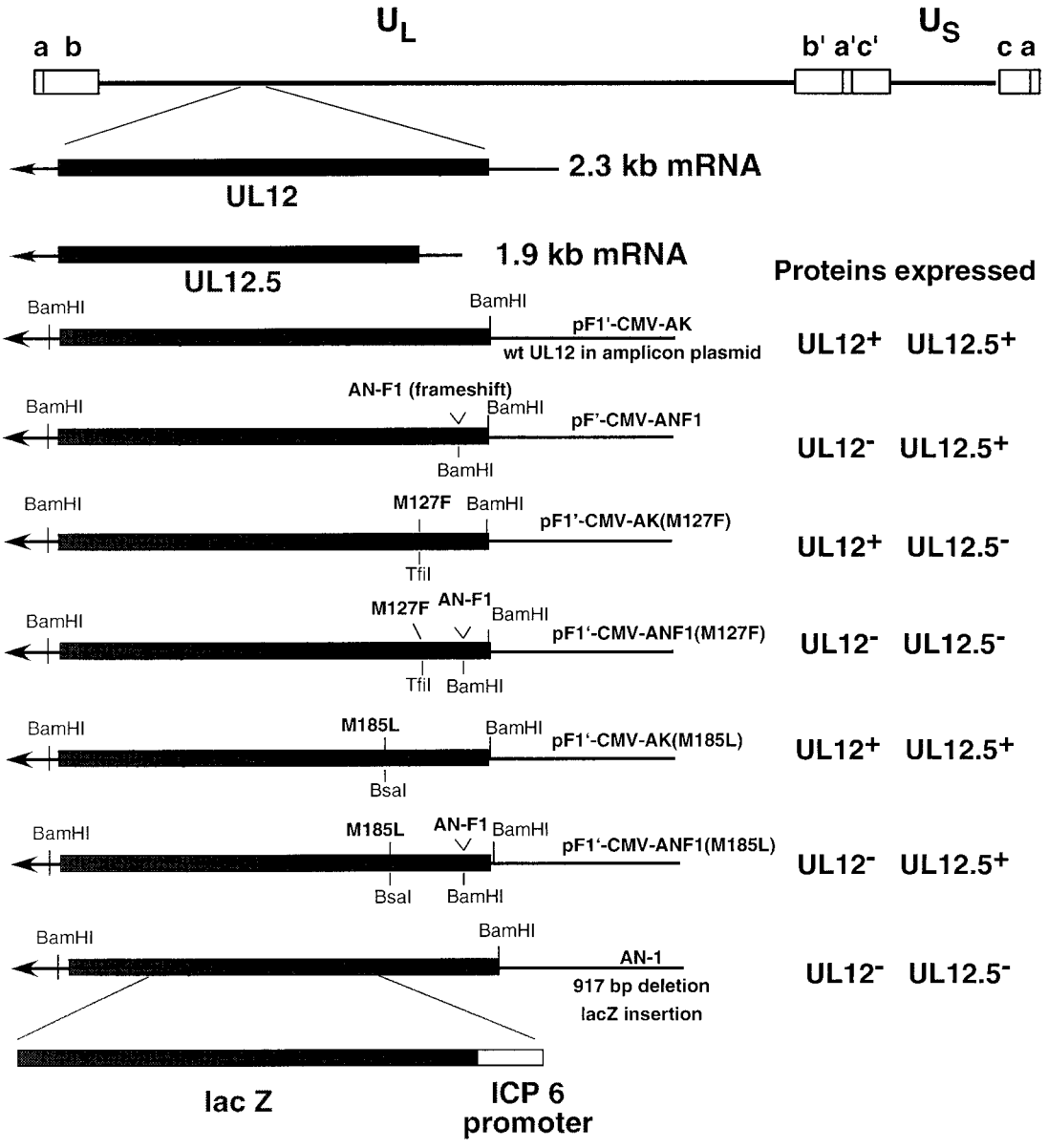


FIG. 1. The HSV-1 genome showing the positions of the 2.3- and 1.9-kb mRNAs encoding full-length UL12 and the N-terminally truncated UL12.5, respectively, are shown. The genomic coordinates of the UL12 gene are 25011–26889 (McGeoch *et al.,* 1986a). Below the UL12 and UL12.5 genes, diagrams of the UL12 expression plasmids used in this study are shown. UL12 was cloned into the amplicon expression plasmid pF1′, in which full-length UL12 expression is driven from the CMV promoter. UL12.5 gene expression would presumably be driven from the endogenous promoter for the 1.9-kb mRNA. pF1′-CMV-AK can express both UL12 and UL12.5. Mutant versions of pF1′-CMV-AK are shown below. The 2-bp insertion resulting in a frameshift mutant introduces a new *Bam*HI site in the ANF1 mutation; the point mutation leading to the substitution of M127 with phenylalanine introduces a *Tfi*I site and the M185 substitution to leucine introduces a *Bsa*I site. Various combinations of double mutant plasmids were constructed. The data showing protein expression from these clones is shown in Fig. 2 and is summarized on the right side of this figure. On the bottom line, the AN-1 deletion–insertion mutation is shown.

*et al.,* 1983; Draper *et al.,* 1986). In addition, the phenotype of the AN-F1 ($UL12^{-}UL12.5^{+}$) viral mutant was identical to that of AN-1 ($UL12^{-}UL12.5^{-}$) mutant, suggesting that UL12.5 cannot compensate for the loss of UL12 (Martinez *et al.,* 1996b). It is still not known whether UL12.5 plays an additional function during lytic growth.

Both the UL12.5 protein and its 1.9-kb message are expressed with early or $\beta$ kinetics (Bronstein *et al.,* 1997; Draper *et al.,* 1986). The UL12.5 protein is present in significantly lower abundance than the UL12 protein during infection (Bronstein *et al.,* 1997), which is consistent with the threefold lower expression of the 1.9-kb transcript relative to the full-length 2.3-kb transcript (Draper *et al.,* 1986). In cells infected with AN-F1, the UL12.5 protein is completely insoluble; however, solubilized extracts from infected cells demonstrate that UL12.5 retains enzymatic activity (Bronstein *et al.,* 1997). Consistent with this result, it has been shown that an expression con-

struct of UL12 lacking the N-terminal 127 amino acids retains nuclease activity (Henderson *et al.,* 1998). Bronstein *et al.* (1997) demonstrated that UL12.5 is associated with both intranuclear capsids and virions, indicating that it may play an important role in infection.

In this article, we map the initiation codon for the UL12.5 gene product and introduce a conservative change at this codon which eliminates UL12.5 without altering the expression of full-length UL12. The resulting mutant, M127F, has allowed us to analyze the role of the UL12.5 protein during infection. Sucrose sedimentation analysis of capsids from cells infected with wild-type or mutant viruses was performed to analyze the association of UL12.5 with viral capsids. We conclude that UL12.5 is not essential for lytic growth and that the apparent association of UL12.5 with viral capsids may be the result of protein aggregation rather than specific association.

## RESULTS

### Mapping the initiation codon for the UL12.5 gene product

To determine the requirement for UL12.5 expression during HSV-1 replication, we decided to alter the UL12.5 start codon with a conservative amino acid change. Such a mutation should have little effect upon UL12 activity, while eliminating UL12.5 translation. The UL12.5 transcript has been mapped (Costa *et al.,* 1983), and we considered the in-frame methionines at positions 127 and 185 as being potential candidates for the start site of the UL12.5 gene product based on the size of the predicted ORF and codon start site preferences. In-frame methionines are also present at 215, 274, 328, 334, 376, and 390. We mutated M127 to a phenylalanine, since sequence alignment data revealed a phenylalanine at this position in many of the UL12 homologs in other herpesviruses. M185 was substituted with a leucine. These substitutions were constructed in the amplicon-based CMV promoter expression plasmid (F′-CMV series) (Fig. 1) (see Materials and Methods).

### Expression by transfection

The ability of clones bearing the M127F and M185L mutations to express UL12 and UL12.5 was tested in both infected and transfected cells expressing wild-type and mutant versions of UL12 and UL12.5 by Western blot analysis using a polyclonal antibody raised against the entire UL12 protein expressed in *Escherichia coli*. Although the UL12 gene is predicted to encode a protein with a molecular weight of 67 kDa, full-length UL12 protein migrates at 85 kDa. The difference between the predicted and apparent molecular weights has been explained by the presence of a proline-rich N-terminus. Infected cells are shown on the right-hand portion of Fig. 2 (lanes 7–10). In cells infected with wild-type KOS, a

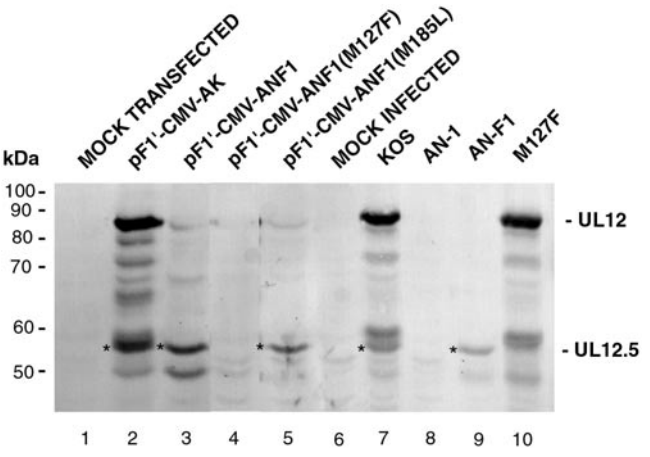


FIG. 2. Western blot of extracts of cells infected mutant viruses or transiently transfected with various wild-type and mutant expression plasmids. Vero cells were mock transfected (lane 1) or transfected with 2 $\mu$g of the following plasmids: pF1′-CMV-AK (lane 2); pF1′-CMV-ANF1 (lane 3); pF1′-CMV-ANF1(M127F) (lane 4); or pF1′-CMV-ANF1(M185L) (lane 5). Vero cells were infected with each of the following viruses at an m.o.i. of 3 PFU/cell: lane 6, mock; lane 7, KOS; lane 8, AN-1; lane 9, AN-F1; lane 10, M127F. At 24 h posttransfection or 16 h postinfection cells were lysed, and extracts subjected to electrophoresis on an 12% SDS–polyacrylamide gel. Following Western blotting, UL12 gene products were detected with antibody BWp12. The position of the UL12.5 gene product is indicated by an asterisk. Molecular weight markers are shown on the left.

full-length UL12 protein at 85 kDa is apparent as well as the UL12.5 protein at 55 kDa (marked with an asterisk) (Fig. 2, lane 7). As expected, cells infected with AN-1 exhibit neither band (Fig. 2, lane 8). As previously reported, cells infected with AN-F1 exhibit only the 55-kDa UL12.5 band (Fig. 2, lane 9). Cells infected with a viral mutant bearing the M127F mutation (described below) exhibit the full-length UL12 product at 85 kDa; however, no UL12.5 gene product is present, despite the presence of bands migrating just above the UL12.5 band (lane 10). These bands likely represent degradation products of full-length UL12, since they are only seen in cells which are capable of producing the full-length protein. The M127F extract contains at least two degradation products migrating near UL12.5, but does not appear to contain the UL12.5 band. Because the presence of the potential breakdown products complicates the analysis of whether the M127F virus produces UL12.5, we analyzed cells transiently transfected with amplicon-expression plasmids bearing wild-type (pF1′-CMV-AK) and mutant forms of the UL12 gene (Fig. 2, lanes 2–5). As expected, mock-transfected cells exhibited no staining (Fig. 2, lane 1); however, cells transfected with the wild-type expression clone contain major species of 85 and 55 kDa. In these transfected cells, even more putative degradation products with intermediate mobilities were observed (Fig. 2, lane 2). Addition of protease inhibitors did not significantly reduce the amount of degradation product (data not shown). In contrast, an amplicon plasmid de-

signed to express the frameshift mutation AN-F1 exhibited only the 55 kDa and a faster migrating band (Fig. 2, lane 3), supporting the notion that the intermediate bands represent degradation products of the full-length UL12. To determine whether the methionine mutants are capable of expressing the UL12.5 band, we moved both the M127F and the M185L mutations into the AN-F1 mutant background which cannot express the full-length UL12 protein. These constructs would not make full-length UL12 and would, therefore, not contain degradation products. In cells transfected with the AN-F1/M127F double-mutant amplicon, neither the full-length nor the UL12.5 band is present (Fig. 2, lane 4). In cells transfected with the AN-F1/M185L double-mutant amplicon, only the 55-kDa UL12.5 band is apparent, as would be expected if it were still able to synthesize UL12.5 (Fig. 2, lane 5). Therefore, we conclude that the M127F mutation abrogates the synthesis of UL12.5. It is formally possible that proteolytic breakdown products of UL12 may substitute and play a role in the viral life cycle analogous to the UL12.5 protein. For reasons described below, we favor the notion that the proteolytic breakdown occurs during the lysis of cells and that the bands which migrate at positions near to UL12.5 do not play a role in HSV replication.

### Generation of the M127F mutant virus

The M127F mutation was introduced into the viral genome by marker transfer, as described under Materials and Methods. The progeny from three independent transfections were triple-plaque-purified. To confirm the introduction of the desired mutation, DNA from each of the viral stocks was digested with *Tfi*I, subjected to gel electrophoresis, and hybridized to a radiolabeled UL12 probe. The introduction of a novel *Tfi*I site resulted in the loss of a 2.3-kb UL12-containing fragment, a gain of 1.9 kb, and 0.4-kb fragments in all three mutants (data not shown). To confirm that the desired mutation was present, DNA from one of the recombinant viruses (M127F-1) was amplified by PCR, subcloned, and sequenced with the L127 primer (5′-TCCGCGGTCCAC-CATAC-3′) using a Sequenase kit (U.S. Biochemicals, Cleveland, OH). No additional mutations were found, and M127F-1 was used in subsequent experiments (data not shown).

### Growth properties of the M127F mutant virus

Next we asked whether the inability to express the UL12.5 gene product resulted in a growth defect. To measure the efficiency of virus production, Vero cells were infected with KOS, M127F, AN1, or ANF-1 at an m.o.i. of 1 PFU/cell and harvested at various times up to 48 h postinfection (Fig. 3). The growth curve shown in Fig. 3 indicates that the M127F virus grows as well as wild-type; whereas, as previously reported, AN-1 and

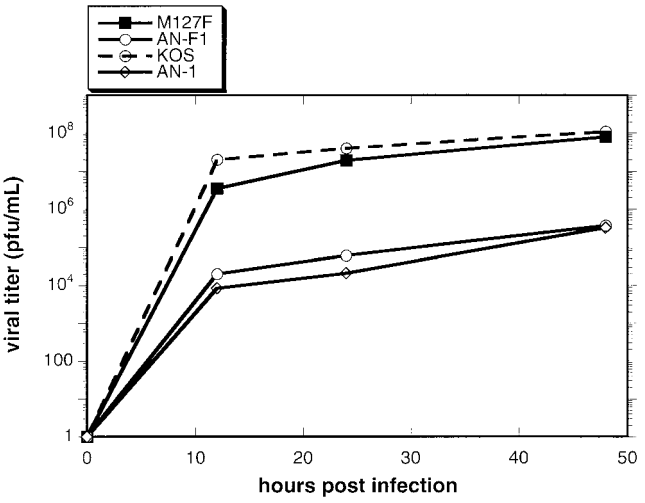


FIG. 3. Growth curve of KOS and alkaline nuclease mutants. Separate plates of Vero cells were infected with either KOS (– – ○ – –), AN-1 (— ◇ —), AN-F1 (— ○ —), or M127F (— ● —) at an m.o.i. of 1 PFU/cell. Time points were harvested at 12, 24, and 48 hpi. Total viral yield was determined by titration on permissive 6-5 cells.

AN-F1 were severely compromised for virus production, exhibiting a two to three log reduction in yields compared to wild-type KOS and M127F. Therefore, the inability to express UL12.5 did not impair viral growth. To confirm this result, transient complementation experiments were performed in which Vero cells were transfected with amplicon-based expression plasmids and superinfected with AN-1. The viral progeny were titered on complementing 6-5 cells. In this type of transient complementation test, plasmids bearing the wild-type version of UL12, M127F, and M185L were able to complement the null mutant, whereas plasmids bearing the AN-F1 mutation were not (data not shown). Thus we can conclude that UL12.5 is not essential for viral growth.

### UL12.5 in capsids

In previous experiments, UL12.5 was found entirely in the insoluble fraction of KOS-infected cell lysates, whereas UL12 was almost entirely soluble (Bronstein *et al.,* 1997). Sucrose gradient analysis of capsids isolated from infected cells indicated UL12.5 was found associated with virions and capsid fractions. This result was interpreted to indicate that the product of the UL12.5 gene is a capsid-associated nuclease which may play a role in processing of genomic DNA during encapsidation. In this article, capsids were isolated from KOS- and various mutant-infected cells and subjected to sucrose gradient centrifugation and fractions examined for the presence of UL12 and UL12.5 by Western blot analysis. The presence of the major capsid protein, VP5, was used to indicate which fractions contained A, B, and C capsids (Fig. 4A). In cells infected with KOS, both UL12 and UL12.5 were detected throughout the gradient, whereas

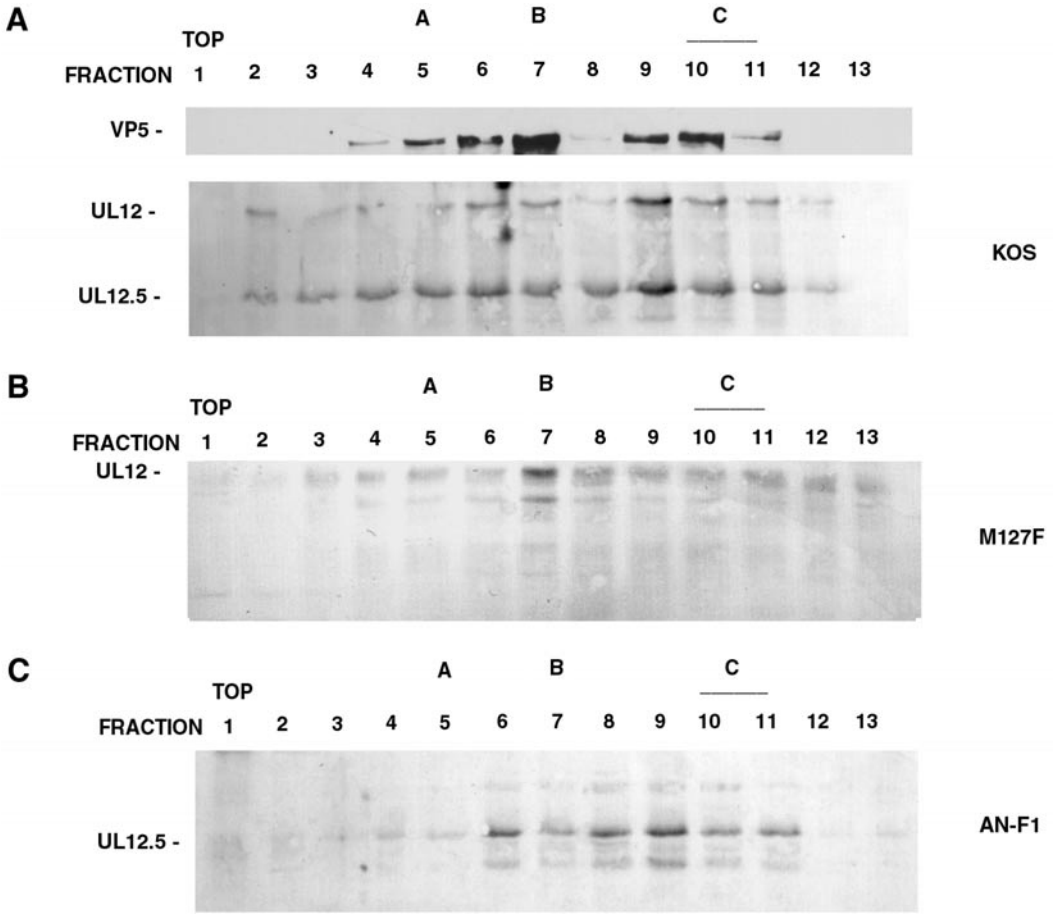


FIG. 4. Sucrose gradient analysis of capsids from KOS- or alkaline nuclease mutant-infected cells. Infected cell lysates were fractionated by ultracentrifugation on a 15–50% (w/v) sucrose gradient as described under Materials and Methods. Thirteen fractions were collected and examined by ECL immunoblotting (for VP5 protein) or by alkaline phosphatase immunoblotting (for UL12 and UL12.5 proteins). The positions of A, B, and C capsids as determined by VP5 position are marked. (A) KOS-infected cells; (B) M127F-infected cells; (C) AN-F1-infected cells.

VP5 is seen predominantly in fractions known to contain A, B, and C capsids (Fig. 4A). In cells infected with the M127 virus, the UL12 protein is seen across the gradient, but no bands in the vicinity of UL12.5 are seen (Fig. 4B). In cells infected with AN-F1 (UL12−, UL12.5+) no UL12 is seen, and UL12.5 is seen in several fractions across the gradient (Fig. 4C). In these experiments, it was difficult to determine whether the UL12.5 was specifically associated with capsid fractions or was present across the gradient as a result of nonspecific aggregation. To definitively answer this question, we analyzed capsids from cells infected with a *ts* mutant (tsG8) in the major capsid protein, shown to be incapable of forming A, B, or C capsids (Schaffer *et al.,* 1973; Weller *et al.,* 1987).

Figure 5 shows that in cells infected with KOS or *ts*G8 (1178) at 34°C, capsid forms are seen in the expected fractions, while UL12.5 is present across the gradient. Similar results were obtained in cells infected with KOS at 39.5°C (Fig. 6A) except that UL12.5 is not distributed as broadly across the gradient. On the other hand, in cells infected with *ts*G8 at 39.5°C (Fig. 6B), no capsids were observed; furthermore, no VP5 was associated with

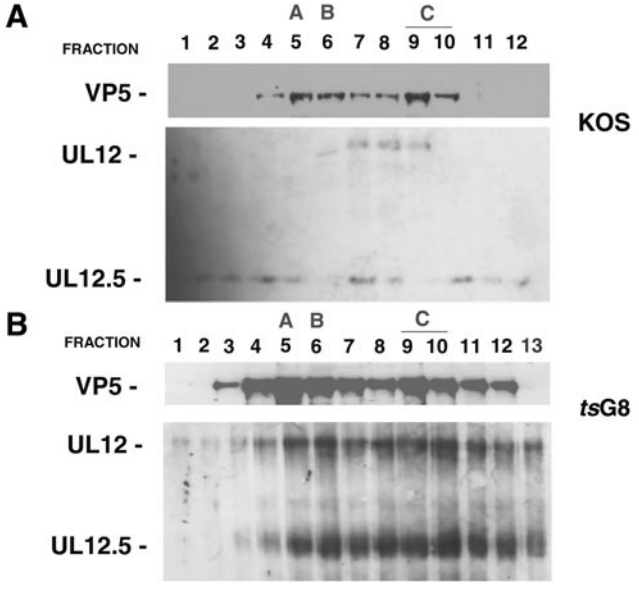


FIG. 5. Sucrose gradient analysis of lysates from KOS- or *ts*G8-infected cells at 34°. Capsids were isolated and fractions collected as described under Materials and Methods and examined by ECL immunoblotting for both VP5 and UL12 and UL12.5 proteins. In B, the UL12 and UL12.5 bands migrated in a broader band than normally seen.

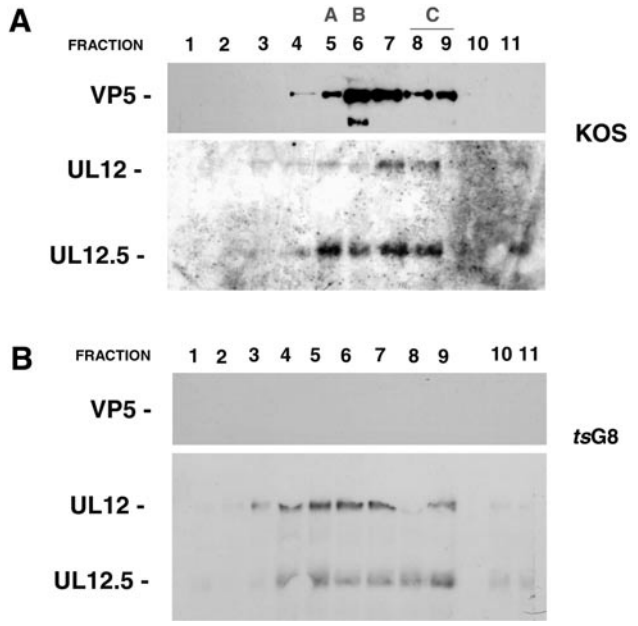


FIG. 6. Sucrose gradient analysis of lysates from KOS- or *ts*G8-infected cells at 39° (nonpermissive temperature). Capsids were isolated and fractions collected as described under Materials and Methods and examined by ECL immunoblotting for both VP5 and UL12 and UL12.5 proteins. Exposures were approximately 3 min, except for VP5 panel of (B), which was exposed for 30 min to emphasize that no VP5 was present in the gradient fractions.

any gradient fraction expected to contain capsids. UL12 and UL12.5 were still seen in fractions throughout the gradient. Thus although the position of UL12.5 in some gradients appears to migrate with capsid-containing fractions (Figs. 4C and 6A), in other gradients UL12 and UL12.5 are distributed more widely across the gradient with no apparent correlation to position of capsids (Figs. 4A and 5B). The most convincing evidence, however, that UL12 and UL12.5 are not specifically associated with capsids comes from the distribution of UL12 and UL12.5 in gradients which do not contain capsids at all (Fig. 6B).

## DISCUSSION

Alkaline nuclease is well conserved throughout the herpesvirus family, suggesting that it plays an important role in the life cycle of herpesviruses. The growth properties of the null and other mutants of alkaline nuclease confirm that it is essential for efficient production of progeny virus (deWind *et al.,* 1994; Gao *et al.,* 1998; Martinez *et al.,* 1996a; Patel *et al.,* 1996; Weller *et al.,* 1990). The major defect in UL12 null mutant infections is manifested at the point of capsid egress from the nucleus; in the absence of UL12, few if any DNA-containing capsids appear in the cytoplasm (Shao *et al.,* 1993). We have previously proposed that the defect in egress is due to the presence of abnormalities in the viral DNA genome which in turn affects the stability of DNA-containing capsids. We and others have reported that HSV-1 DNA replication intermediates have a complex branched structure (Martinez *et al.,* 1996a; Severini *et al.,* 1994, 1996); furthermore, the DNA which accumulates in cells infected with UL12 null mutants exhibits an altered structure compared to DNA in cells infected with wild-type viruses (Martinez *et al.,* 1996a). We have recently reported that UL12 in conjunction with the major single-strand binding protein, ICP8, can carry out a strand exchange reaction *in vitro* (Reuven *et al.,* 2002). This result is consistent with our previous proposal that UL12 plays a role in DNA recombination and/or genome maturation (Goldstein and Weller, 1998b).

In any model of UL12 function, it is important to determine which viral gene products are responsible for various activities and even more importantly are required for lytic viral growth. The UL12 gene has an unusual structure, in that a subgenic mRNA is expressed which encodes an N-terminally truncated viral gene product designated UL12.5. Alkaline nuclease is well conserved among all members of the herpesvirus family for which sequence information is available: UL12 homologues all contain seven conserved motifs (Goldstein and Weller, 1998a; Martinez *et al.,* 1996b). Interestingly, HSV-1 and HSV-2 contain approximately 120 amino acids at their N-terminus, which are not shared with homologues from the other herpesviruses (Martinez *et al.,* 1996b). In fact, for the HSV-1 gene the first conserved region, motif I, is located at residue 217, leading to the suggestion that UL12.5 may encode an enzymatically active form of the enzyme. This suggestion was confirmed by Bronstein *et al.,* who showed that UL12.5 isolated from cells infected with ANF1 which only express UL12.5 retains endo- and exonuclease activities (Bronstein *et al.,* 1997). Additional biochemical characterization revealed that UL12.5 protein was similar to full-length UL12 with respect to pH optimum, ionic strength, and divalent cation requirements. Bronstein *et al.* demonstrated that UL12.5 appeared to be specially associated with both intranuclear capsids and virions (Bronstein *et al.,* 1997). The finding that the UL12.5 protein represents a capsid-associated form of AN which exhibits nucleolytic activity suggests that it may play some role in the processing of genomic DNA during encapsidation. We previously showed that expression of UL12.5 could not relieve the growth defects of a UL12 null mutant virus (Martinez *et al.,* 1996b), but the question of whether UL12.5 by itself is essential for lytic viral growth was not addressed in previous studies on UL12.5. Furthermore, the start codon of UL12.5 had not been mapped.

In the present study, the start site of the UL12.5 was mapped to Methionine 127 and a mutation in which this methionine was changed to phenylalanine was constructed and introduced into the viral genome. The mutant M127F exhibits wild-type growth properties in Vero cells, suggesting that UL12.5 is not essential for lytic viral

growth. A formal possibility exists, however, that UL12.5 or a protein of a similar size as UL12.5 is essential and that in our experiments, proteolytic fragments derived from full-length UL12 may substitute for the loss of UL12.5 in the M127F mutant. While we cannot rule out this possibility, we have shown that the sucrose gradient fractions from cells infected with M127F do not contain detectable UL12.5-related fragments. Thus, we can conclude that such proteolytic fragments do not appear to associate with capsids or even throughout the gradient as bone fide UL12.5 does when it is expressed. It is more likely that the fragments seen in extracts are a result of proteolysis which occurs during or after cell lysis and that they are not actually present *in vivo* in infected cells. In summary we conclude that UL12.5 is not essential for viral lytic growth.

Several examples of similar in-frame truncation products encoded independently of their full-length primary products have been detected in the HSV-1 genome. The US1.5 gene is an independently transcribed and translated truncated version of the US1 (ICP22) gene (Carter and Roizman, 1996a,b); the UL8.5 gene appears to encode an N-terminally truncated version of UL9 (Baradaran *et al.,* 1994, 1996). The UL15.5 gene is independently expressed from the UL15 gene (Baines *et al.,* 1997; Yu *et al.,* 1997; Yu and Weller, 1998a,b); and the UL26.5 gene is expressed from an independent mRNA within the UL26 gene (Liu and Roizman, 1991; Matusick-Kumar *et al.,* 1994). For UL1.5 and UL8.5 it is not known whether the truncated versions of the genes play a role in viral infection distinct from the full-length products. The UL15.5 gene product has been shown to be dispensable for viral growth (Yu and Weller, 1998a), while the UL26.5 protein is known to play a very important role as the scaffold protein during viral assembly. Mutational analysis has indicated that both UL26 and UL26.5 are required for optimal viral growth (Matusick-Kumar *et al.,* 1994). It is not clear why HSV-1 expresses a nonessential protein such as UL12.5. It is possible that UL12.5 plays a role in viral replication in animals but not in cell culture. Alternatively, UL12.5 may be involved in establishing or maintaining latent infection. It is also possible that UL12.5 is a pseudogene, perhaps a remnant of the process leading to the evolution of the full-length UL12 gene and is therefore no longer necessary. As mentioned above, the gene structure of UL12 is unusual in that the N-terminus of both HSV-1 and HSV-2 UL12 is approximately 120 amino acids longer than that of all other UL12 homologues. Thus it is possible that at some point during evolution, an additional function was added to the UL12 protein, creating the full-length version of UL12. Thus, the continued expression of UL12.5 may be superfluous.

Previous work with UL12.5 indicates that it is completely insoluble in infected cells and may be associated with both intranuclear capsids and virions (Bronstein *et al.,* 1997). Furthermore, virions treated with guanidine hydrochloride (so-called G capsids) also retained some UL12.5, indicating a close association. In this study, sucrose sedimentation analysis of capsids from cells infected with wild-type or mutant viruses indicates that both UL12 and UL12.5 are found in fractions from across the sucrose gradient, which do not always correlate with the presence of viral capsids. Furthermore, UL12.5 is found in fractions across the gradient even in cells infected under conditions in which no capsids are formed. These results indicate that UL12.5 does not specifically associate with viral capsids. The most likely explanation for the appearance of UL12.5 across the gradient may be aggregation, as UL12.5 is known to be insoluble in infected cell lysates. The previous observation that UL12.5 is seen in G capsids may reflect a nonspecific association with capsids which are present in some of the same gradient fractions. We cannot rule out however the possibility that some UL12.5 is incorporated into capsids; however, it seems unlikely that it plays an important role in lytic viral infection.

## MATERIALS AND METHODS

### Cells and viruses

African green monkey kidney cells (Vero; American Type Culture Collection, Rockville, MD) were propagated as described previously (Weller *et al.,* 1983). The S22 and 6-5 cell lines, which are permissive for UL12 mutants, were previously described (Carmichael *et al.,* 1988; Shao *et al.,* 1993). The KOS strain of HSV-1 was used as the wild-type virus. The UL12 null mutant virus AN-1 ($UL12^{-}UL12.5^{-}$) and the frameshift mutant virus AN-F1 ($UL12^{-}UL12.5^{+}$) were previously described (Martinez *et al.,* 1996b; Weller *et al.,* 1990) (see Fig. 1). TsG8 (ts1178), a temperature-sensitive mutation in the UL19 (VP5 gene), was previously described (Schaffer *et al.,* 1973; Weller *et al.,* 1987).

### Plasmids

The plasmid pAK, containing the UL12 gene and its promoter, was described in Martinez *et al.,* 1996b. The amplicon plasmid pF1′-CMV containing the HSV-1 $ori_S$ sequence and the "a" sequence was generously provided by Ann D. Kwong (Hong *et al.,* 1996). The plasmid pF1′-CMV-AK in which the UL12 gene is expressed from the CMV promoter was described in (Goldstein and Weller, 1998a). To generate pUC119-BD2+2, the UL12-containing *Eco*RI–*Hin*dIII fragment from M13-BD2+2 (described in Martinez *et al.,* 1996b) was cloned into the *Eco*RI and *Hin*dIII sites of pUC119. To generate pANF1, the UL12-containing region of M13-BD2+2 was PCR amplified using primers U127 (5′-AGGCGATACTGTCGTCGGTGGC-3′) and L127 (5′-TCCGCGGTCCACCATAC-3′). The product was digested with *Afl*II and *Xho*I and subcloned into pAK, replacing the corresponding fragment,

to produce pANF1. The plasmid pANF1 was sequenced to ensure that no secondary mutations were introduced by the PCR process. To generate plasmid pF1′-CMV-ANF1, a series of subcloning steps were required, as the ANF1 frameshift mutation is located in close proximity to the UL12 start site (which, in plasmid pF1′-CMV-AK, contains an artificially introduced *Bam*HI site). First, the ends of a 0.7-kb *Xho*I fragment from pF1′-CMV-AK were filled in with Klenow enzyme, and this fragment was ligated into the *Sma*I site of pUC119 to generate pUC-AK/*Xho*. To generate pANF1/*Xho*, a 0.5-kb *Dsa*I fragment from M13-BD2+2 was used to replace the 0.5-kb *Dsa*I fragment in pUC-AK/*Xho*. Finally, to generate pF1′-CMV-ANF1, a 0.7-kb *Xho*I fragment of pANF1/*Xho* was ligated into pF1′-CMV-AK, replacing the corresponding *Xho*I fragment. See Fig. 1 for a diagram of single and double mutations in the pF1′-CMV-AK background.

### Construction of point mutations

Mutations were constructed by two-step PCR (Ausubel *et al.,* 1997). Primers were obtained from National Biosciences, Inc. (Plymouth, MN). The outside primers were AlaC (described in Goldstein and Weller, 1998a) and a M13–24mer (5′ AACGCCAGGGTT TTCCCAGTCACGAC-3′). The mutagenic primers for making the M127 to F mutation (ATG to TTC) were as follows, with the mutated nucleotides bold and underlined: M127F-T (5′-coordinate number 26,520-CTTGA**T**TCT**T**T**C**TGGTCGGCGTCGGT-GATCCC-3′), M127F-B (5′-coordinate number 26,501-CCGACCA**G**A**A**AGA**A**TCAAGGTCCGGGGAGT-3′). M127F was constructed with the M127F primers, the mutated PCR product was digested with *Afl*II and *Xho*I, and the 0.6-kb fragment subcloned into pAK to generate pAK(M127F). The clone was confirmed by the introduction of a novel *Tfi*I site and sequenced to ensure that the PCR process did not generate any secondary mutations. Then, to generate pF1′-CMV-AK(M127F) and pF1′-CMV-ANF1(M127F), a 1-kb *Eco* 72I/*Bsi* WI fragment from pAK(M127F) was ligated into pF1′-CMV-AK and pF1′-CMV-ANF1, respectively, replacing the corresponding fragments.

To generate pF1′-CMV-AK(M185L), two-step PCR was performed as described above. The mutagenic primers for the M185 to L mutation (ATG to CTC) were M185-upper (5′- coordinate number 26,347 -GGAGGCGGGT**C**T **C**GTGGACCGCGG ACTCGGC-3′) and M185-lower (5′-coordinate number - 26,317-CCGAGTCCGCGG TCCAC-**G**A**G**ACCC GCCTCCT-3′). These substitutions generate a novel *Bsa*I site. The PCR product was digested with *Msc*I and *Bsr*GI, and the 0.3-kb fragment was subcloned into pF1′-CMV-AK, replacing the corresponding fragment in pF1′-CMV-AK. Introduction of the desired mutation was confirmed by the presence of the novel *Bsa*I site. Thus both the M127F and the M185L mutations were cloned into the amplicon vector, pF1′-CMV, such that the expression of the full-length UL12 will be driven by the CMV promoter. It is likely that UL12.5 expression will be under control of its endogenous promoter in these constructs.

### Marker transfer

To generate a recombinant virus containing the M127F mutation, a 3.2-kb *Sph*I fragment from plasmid pAK(M127F) was cotransfected with infectious AN-1 DNA into 6-5 cells as described (Martinez *et al.,* 1996b). AN-1 makes blue plaques when plated in the presence of X-gal due to the presence of the *lac*Z gene insertion. White plaques were selected, and three plaques from independent transfections were triple-plaque-purified. Introduction of the mutation, with its novel *Tfi*I site, was confirmed by Southern blotting (data not shown).

### Western blotting

UL12 gene products were detected from lysates of both transfected cells and infected cells as follows. Vero cells ($1 \times 10^6$) were plated onto 60-mm dishes and either transfected with 2 $\mu$g of the various plasmid clones using Lipofectamine (Life Technologies, Grand Island, NY) or infected at an m.o.i. of 3 with various viral mutants. Twenty-four hours posttransfection or 16 h postinfection, the cells were scraped from the plates and pelleted in a Beckman TJ-6 centrifuge at 2000 rpm for 10 min. Cell pellets were rinsed in PBS and resuspended in 350 $\mu$l 1× SDS–PAGE loading buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 5% $\beta$-ME, 10% glycerol, and 0.1% bromphenol blue). Lysates in loading buffer were boiled for 5 min and 50-$\mu$l aliquots were subjected to electrophoresis on 12% SDS–polyacrylamide gels. Protein was blotted onto Immobilon P membranes (Millipore, Bedford, MA) and visualized by alkaline phosphatase reaction (Promega, Madison, WI). Polyclonal antibody Bwp12, prepared against the entire UL12 protein expressed in *E. coli*, was kindly provided by Peter Weber (Parke-Davis, Ann Arbor, MI) (Bronstein and Weber, 1996). Bwp12 was used as the primary antibody at a dilution of 1:10,000 at room temperature for 1 h.

### Capsid isolation by sucrose gradient centrifugation

Five 162-cm$^2$ flasks of cells were infected at an m.o.i. of 5 for 18 h, removed by scraping, and pelleted in a Beckman TJ-6 centrifuge at 2000 rpm for 15 min. The pellets were rinsed in PBS and lysed by resuspension in 2 ml PBS to which was added 2 ml of 2× lysis buffer containing 2% Triton X-100, 1 M NaCl, 2 mM EDTA, 40 mM Tris pH 7.6, and protease inhibitors [one complete tablet of protease inhibitor cocktail (Roche, Indianapolis, IN) per 10 mL, 10 $\mu$g pepstatin (Sigma, St. Louis, MO) per mL, and 300 $\mu$l liquid protease inhibitor cocktail for mammalian cell extracts (Sigma) per 10 mL]. Cells were freeze/thawed three times and disrupted by sonication

using a Misonix XL (Farmingdale, NY) fitted with a bath attachment. The lysate was precleared by centrifugation at 8000 rpm at 4°C for 30 min in an SW41 rotor. Capsids were partially purified by pelleting through a cushion of 35% (w/v) sucrose in TNE (500 mM NaCl, 20 mM Tris pH 7.6, 1 mM EDTA) by centrifugation at 24,500 rpm at 4°C for 60 min in an SW41 rotor. Capsid pellets were suspended in TNE and the capsids purified on 20–50% (wt/vol) sucrose gradients produced on a BioComp Gradient Master (Fredericton, N.B., Canada), by centrifugation at 24,500 rpm at 4°C for 60 min in an SW41 rotor. Capsid bands were visualized by light scattering, and fractions of the gradients were collected with a BioComp Piston Gradient Fractionator, Model 151. Proteins in the fractions were precipitated with TCA on ice for 10 min, pelleted, resuspended in 1× SDS–PAGE loading buffer as described above, and boiled for 5 min. Aliquots of approximately one-third of the total yield from each fraction were loaded onto duplicate SDS–12% polyacrylamide gels. Proteins were blotted onto Immobilon P membranes as described above. The UL12 gene product was visualized using the Bwp12 antibody with the Immun-star detection system according to manufacturer's instructions (Bio-Rad, Hercules, CA), while the duplicate gel was probed with a monoclonal antibody against the major capsid protein VP5 (ABI, Columbia, MD). VP5 was visualized using ECL (enhanced chemiluminescence) according to the manufacturer's instructions (Amersham, Piscataway, NJ).

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